Vietnam Journal of Science and Technology ***56*** *(2C) (2018) 16-22*

FECAL CONTAMINATION AND THE PROPORTION OF HUMAN-ASSOCIATED *E. coli* ALONG NHUE RIVER, VIET NAM

Mai Tanaka[1], Hidenori Harada[1, *], Shigeo Fujii[1], Min Li Chua[1], Nguyen Duy Hung[2] Nguyen Pham Hong Lien[2], Nghiem Trung Dung[2], Ryota Gomi[3]

[1]*Graduate School of Global Environmental Studies, Kyoto University, Yoshida-honmachi, Sakyo-ku, Kyoto 606-8501 JAPAN*

[2]*School of Environmental Science and Technology, Hanoi University of Science and Technology, 1 Dai Co Viet Road, Ha Noi, Viet Nam*

[3]*Graduate School of Engineering, Kyoto University, Kyoto daigaku-katsura, Nishikyo-ku, Kyoto 615-8530 JAPAN*

[*]Email*: harada.hidenori.8v@kyoto-u.ac.jp*

Received: 10 May 2018; Accepted for publication: 20 August 2018

ABSTRACT

According to rapid urbanization in Asian countries, the amount of domestic wastewater discharge has been increasing, resulted in water pollution and potential health risk on human. To secure biological safety of rivers, it is critical to understand the sources of contamination. The present study applied the human-associated *E.coli* genetic marker (H8) to characterize the source of microbial contamination. *E. coli* concentration and other pollutants indices were investigated at 10 sites along the river: S1 (upstream) – S10 (downstream). For 220 *E. coli* isolates collected from river water samples, Real-Time PCR was performed with H8. The results showed that organic pollutants peaked at the middle stream, although they were originally low at upstream (S1). The positive proportion of H8 with *E. coli* peaked at 40.9 % at middle stream (S6), which was significantly higher than S1 (4.5 %) (p = 0.012). This high proportion implied the relative dominance of human-associated *E. coli*, which were in line with a large inflow of sanitary wastewater in middle stream, indicated by land use along the river. Thus, we successfully demonstrated the usefulness of H8 to track the source of microbial contamination in the river.

Keywords: human-associated *E.coli*, Nhue River, Viet Nam, source tracking, genetic marker.

1. INTRODUCTION

Rapid urbanization has been increasing the amount of domestic wastewater generated. The discharge of untreated domestic wastewater causes surface water pollution and potentially threatens human health. To secure biological safety of rivers, it is critical to understand the source of microbial contamination. Microbial source tracking (MST) markers have been recently used to identify the source of microbial contamination by using host-associated genes found in bacteria, protozoa, and viruses from animal feces, as well as human [1, 2]. Previous studies [3,

4] often used biomarkers targeting on *Bacteroides* spp., which are dominant members of the fecal flora and obligate anaerobes. However, it has limitations of which *Bacteroides* markers persist for a short period of time (99 % decay in < 8 days) [5]. Further, *Bacteriodes* spp. are not commonly used as a fecal indicator bacteria in water environment.

Unlike *Bacteroides* spp., *E.coli* has been widely used as one of fecal indicator bacteria. Recently, a human-associated genetic biomarker of *E. coli*, H8, has been developed [6, 7]. Studies conducted in Japan, Australia and Bangladesh had used H8 to check the proportion of human-associated *E.coli* in wastewater and drinking water [6, 7, 8]. However, limited knowledge has been found on the application of H8 to investigate the microbial pollution source in water environment.

Nhue River in Viet Nam runs through most parts of Ha Noi City and it is closely related to human living needs. However, it has been reported that most of the domestic wastewater from urban areas have been discharged directly into the river with high concentration of *E.coli* detected [9]. The present study aimed (1) to characterize microbial contamination of Nhue River by using H8 marker, and (2) to validate the performance of H8 in river water environment as its first application along a river stream.

2. METHODS

2.1. Site description and sample collection

The Day/Nhue River basin (6,965 km^2) is located in Red River Delta [10] (Figure 1*)*. One of the major rivers in this basin is Nhue River, a distributary of Red River that rises in China and discharges into the Gulf of Tonkin. Upstream area of Nhue River is characterized by urban land use, which includes most parts of Ha Noi city, while agricultural activities are practiced in downstream areas, including rice cultivation, livestock and poultry production.

River water samples were collected in three different days between September - November 2017 at 10 sites along Nhue River, in total of 30 water samples (*Figure 1)*: S1 (the intake from Red River) - S10 (downstream). At each of the 10 sampling sites, 500 mL of river water was collected at the center of the main flow. The top 1-2 cm of surface layer were avoided during the collection.

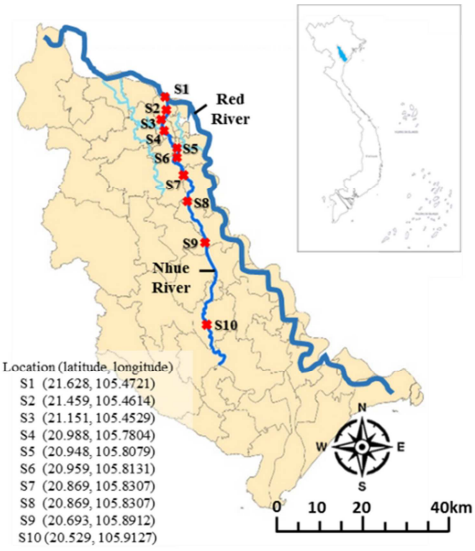

Figure 1. Water sample sites location in the Nhue River basin

2.2. Water quality test

River water quality was analyzed to investigate the organic and fecal contamination levels. At each sampling site, electric conductivity (E.C.) was determined by the Multi Water Quality Checker U-53 (HORIBA). In laboratory, total organic carbon was measured by direct oxidation methods with DR6000 (HACH). In addition, samples were filtered by sterilized and disposable filtration devices (0.45 μm pore size, Microfil V Filtration Device, Fisher Scientific). Then, *E.*

coli were cultivated using a specific enzyme substrate medium (XM-G agar, Nissui) and incubated for 20 ± 2 hours at 37 ^{o}C. Colonies on the medium with blue color profile were enumerated to determine the *E. coli* concentration.

2.3. Land use data collection

The watershed of Nhue River was determined based on ASTER GLOAL DEM date set (USGS and Google Earth satellite images, by using ArcGIS 10.5 and ArcHydro 1.4 (ESRI) [11]. District-level statistics were determined on human, buffalo, chicken, cattle, pig and poultry population density based on provincial statistics [12 - 15].

2.4. Real time PCR assays

In total, 220 environmental *E. coli* isolates were picked up from 30 river water samples and transferred into each well of a 96 well microtiter plate filled with 50 µL *MilliQ* water. Real-time PCR assays were performed on each *E.coli* isolate using H8, human-associated *E.coli* genetic marker. *Table1* describes the primer sets of H8 marker [3]. For the real-time PCR assays, PCR mixture (15 µL) was composed of 4.9 µL of *MilliQ* water, 7.5 µL of QuantiTect SYBR green (Qiagen), 0.3 µL each of forward and reverse primers and 2 µL of *E.coli* samples. All PCR reactions were performed in a 96 well plate for Real Time (Takara, Otsu, Japan) with Thermal Cycler Dice Real Time System 3 (Takara). Positive (DNA from control strains) and negative (sterile water) controls were included for each PCR assay. The Real-time PCR conditions were set at 95^{o}C × 5 min + (95^{o}C × 10 sec + 60^{o}C × 30 sec) × 40 cycles + melting curve analysis [3].

Table1. Primer sets used in this study.

Target Region	Sequence (5'-3')	Product Size (bp)	Gene of Feature
H8	F-ACAGTCAGCGAGATTCTTC R-GAACGTCAGCACCACCAA	177	Sodium / Hydrogen exchanger precursor

2.5. Data analysis

In order to analyze the results from PCR assay, the presence or absence of PCR amplification of each sample were determined by the C_t value of amplification curve. In case that amplification was observed, we determined Tm value based on the results of dissociation curve. The H8-positivity criterias were as such that the C_t value of amplification curve is below 25 cycle and Tm value of dissociation curve showed is within 90 ^{o}C to 93 ^{o}C (Tm value of positive control). In addition, we used Fisher's exact test for count data by using R 3.4.0 (R Core Team, 2017) to check the significant differences among the sampling sites.

3. RESULTS

3.1. Transition of water quality along Nhue River

Figure 2 shows the results of the water quality from upstream to downstream along Nhue River. Despite TOC and E.C. were low at the most upstream and intake from Red River, S1 (TOC: 1.0 mg/L; E.C.: 0.18 mS/cm), they increased gradually along river flow and peaked at

middle stream area, S6 (TOC: 134.0 mg/L; E.C.: 0.76 mS/cm). Then, they decreased gradually along the flow. *E. coli* concentration data also showed the similar trend although its transition was not as clear as TOC and E.C. Thus, together with pollutants indicators, *E. coli* concentration tended to increase from upstream to middle stream, and to decrease gradually towards downstream.

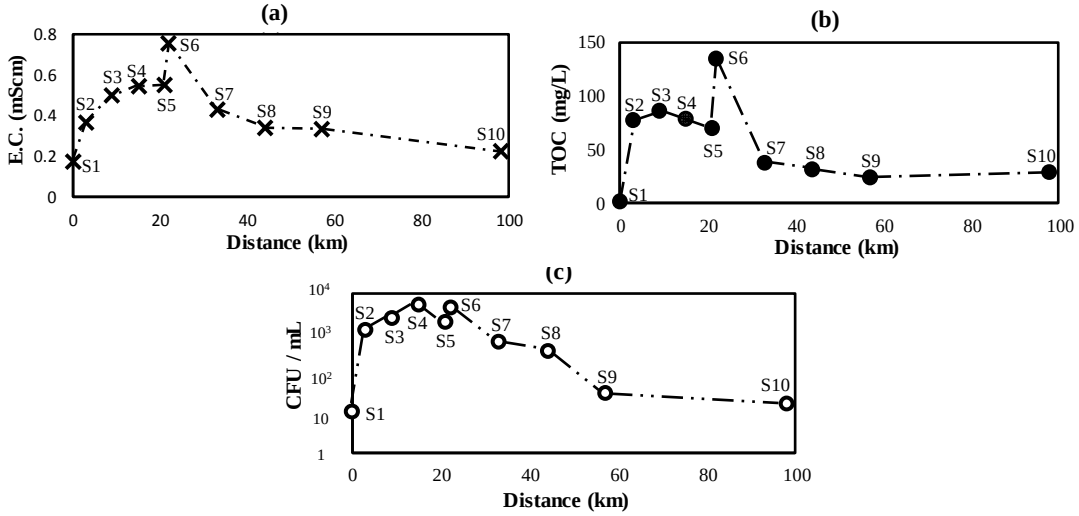

Figure 2. The transition of water quality from upstream to downstream along Nhue River (a) E.C., (b) TOC, (c) *E. coli.*

3.2. Proportions of the H8 in *E.coli* isolates from river water

Figure 3 shows the results of proportions of H8 positive *E. coli* isolates at each sampling site based on the PCR assay. From upstream to middle stream, the proportions continuously increased: 4.5 % (S1), 13.6 % (S2), 13.6 % (S3), 22.7 % (S4), 31.8 % (S5), 40.9 % (S6). Although S7 (13.6 %) showed a sudden decrease, the proportion tended to decrease from middle to downstreams: 31.8 % (S8), 9.1 % (S9) and 9.1 % (S10). We confirm that the proportions of human-derived *E.coli* increased from upstream to middle stream. A significant difference was observed between S1 and S6 ($p = 0.012$). Thus, it was indicated that the H8-positive proportions increased from upstream, peaked at middle stream, and decrease from middle to downstreams.

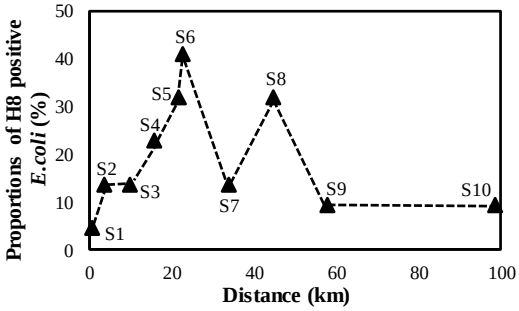

Figure 3 – Proportions of H8 positive *E.coli* isolates along Nhue River.

3.3. Land use data in Nhue River basin

Figure 4 shows the population density of human, poultry and livestock in each district in Nhue River basin. The figures shows that human population density was distinctively high in upstream

to middle streams: all of S1 - S6 were classified into the most populated area (5,001-10,000 cap/km2). In contrast, human population density was low in middle to downstream: all of S7 - S10 (661-2,500 cap/km2). There were no poultry and livestock in up and middle stream areas, including S1 - S6, while their population density were suddenly increased from S7, and kept the high density until downstream. These indicate that sampling sites of S1-S6 were surrounded by human-populated areas and no animal was observed, while sampling sites S7-S10 were surrounded by animal-populated areas.

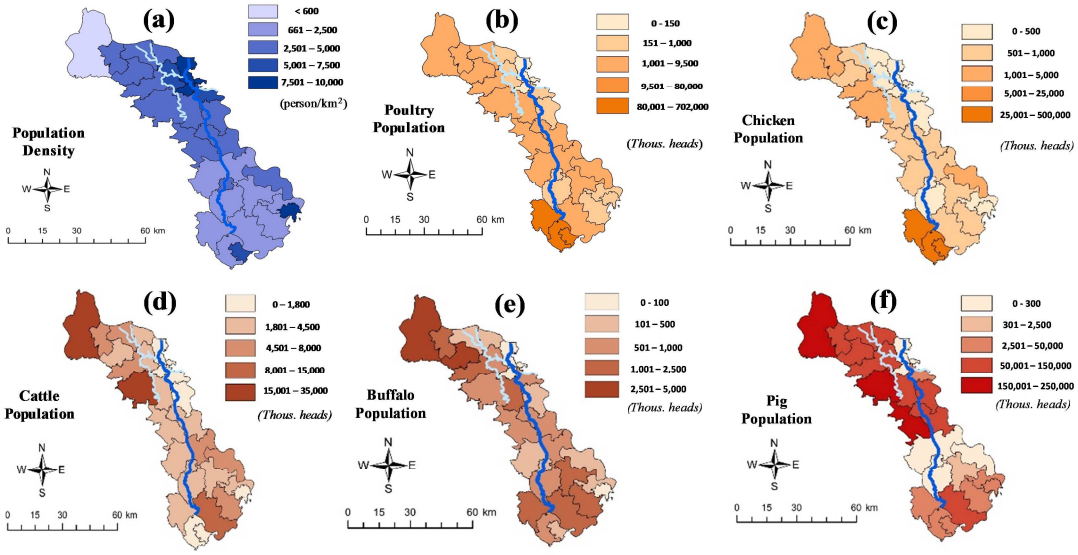

Figure 4. Land use maps in Nhue River basin
(a) Population density, (b) Poultry population, (c) Chicken population,
(d) Cattle population, (e) Buffalo population, (f) Pig population.

4. DISCUSSION

Nhue River in Viet Nam is closely related to the surrounding human societies. However, it has been reported that domestic wastewater has been intensively discharged into the river, with fecal microorganisms including *E. coli* detected at high concentration [9]. The present study also showed that organic pollutants concentration increased and peaked at middle stream (S6) of Nhue River, due to receiving domestic wastewater from To Lich River right before S6. As shown in Figure 4, the areas with high human population were located around up to middle stream of Nhue river. These high concentrations of organic load were likely caused by domestic wastewater from these densely human-populated areas.

Real Time PCR assays with H8 marker showed high positive proportion of *E. coli* with middle stream samples, peaked at 40.9 % at S6, which was significantly higher than S1, the most upstream and intake from Red river (4.5 %). And S8 also showed high positive proportion of H8 marker because there is domestic wastewater canal joining before this point. The sensitivity of H8 marker was investigated in previous studies based on domestic wastewater, which were 30 % and 45 % in Japan [6] and Australia [7], respectively. The highest H8 positive proportion of the present study was similar to these previous studies. These findings imply that most of *E. coli* strains in the present river sample at S6 were derived from human.

As shown in Figure 4, the river flowed through the densely human populated areas. Considering a low coverage of sewage treatment in Ha Noi (33.4 %) [16], the river may had received a great amount of sanitary wastewater between up and middle stream; further, no wastewater inflow derived from livestock and poultry was expected since such animals were not raised around the areas (Figure 4). Thus, the high H8-positive proportion indicating dominance of human-associated *E. coli* indicated in this study are in line with this fact of a large inflow of sanitary wastewater.

However, there were several limitations of H8 marker. Firstly, standard errors were high with H8 positive proportions due to the small sample size at each sampling sites. To find a clear transition of the proportion of human-associated microbial contamination, a large number of samples is required. Secondly, the sensitivity and specificity of H8 marker to human-associated *E. coli* strains would be different with three previous studies carried out in Japan (sensitivity: 30 %, specificity: 99 %) [6], Australia (45 % and 94 %) [7] and Bangladesh (16 %, not confirmed) [8]. No studies on H8 performance were found in Southeast Asia. Further studies are expected to have more proper understanding of the competence between human-associated *E. coli* and nonhuman-associated *E. coli*. Nevertheless, the present study successfully demonstrated the usefulness of the H8 marker to characterize the human source contribution of microbial pollution in river environment. Thus, H8 could be a good option among useful biomarkers to track the source of microbial contamination in river.

Acknowledgment. This study was supported by grants from the Japan Society for the Promotion of Science KAKENHI (no. JP16H04436 and no. JP16H02748). We also would like to acknowledge kind support from Kyoto University – Ha Noi University of Science and Technology Research and Education Program for Environmental studies.

REFERENCES

1. Hughes B, Beale D. J, Dennis P. G, Cook S., Ahmed W. – Cross-comparison of human wastewater-associated molecular markers in relation to fecal indicator bacteria and enteric viruses in recreational beach waters, Public and Environmental Health Microbiology **83** (2017) 1-2
2. Harwood V. J., Staley C., Badgley B. D., Borges K., Korajkic A. – Microbial source tracking markers for detection of fecal contamination in environmental water: relationships between pathogens and human health outcome, Federation in European Microbiological Societies 38 (2014) 1-40.
3. Patricia A. B., Caitlin O. S., Erika T. J., Morgan M, Sandra L. M. – Detection of genetic markers of fecal indicator bacteria in lake Michigan and determination of their relationship to *Escherichia coli* densities using standard microbiological methods, Applied and Environmental Microbiology **71** (2005) 8305-8313.
4. Marek K., Roberto A., Caffaro F., Mayee W., Valerie J. H., Philip M., Roger S. F. – Human-associated *Bacteroides* spp. And human *polyomaviruses* as microbial source tracking markers in Hawaii, Applied and Environmental Microbiology **82** (2016) 6757-6767.
5. Tambalo D. D., Fremaux B., Boa T., Yost C. K. – Persistence of host-associated *Bacteroidales* gene markers and their quantitative detection in an urban and agricultural mixed prairie watershed, Water Research **46** (2012) 2891-2904.

6. Gomi R., Matsuda T., Matsui Y., Yoneda M. – Fecal source tracking in water by next-generation sequencing technologies using host-specific *Escherichia coli* genetic markers, Environmental Science and Technology **48** (2014) 9616-9623.
7. Warish A., Triplett C., Gomi R., Gyawali P., Hodgers L., Toze S. – Assessment of genetic markers for tracking the sources of human wastewater associated *Escherichia coli* in environmental waters, Environmental Science and Technology **49** (2015) 9341-9346.
8. Harada H., Fujimori Y., Gomi R., Ahsan N. Md., Fujii S., Sakai A., Matsuda T. – Pathotyping of *Eschericha coli* isolated from community toilet wastewater and stored drinking water in a slum in Bangladesh, Letters in Applied Microbiology (2018).
9. Takagi A. – Investigation on water environment and pollution load analysis in the Nhue-Day River basin, Viet Nam (2014).
10. ICEM – Day/Nhue river basin pollution sources study: Improving water quality in the Day/Nhue river basin, Viet Nam: Capacity building and pollution sources inventory (2007).
11. U.S. Geological Survey - Earth Explorer. (earthexplorer.usgs.gov/, access on Feb/24/2014).
12. Ha Noi Statistics Office - Ha Noi Statistical Yearbook, GSO Viet Nam, 2016.
13. Hanam Statistics Office – Hanam Statistical Yearbook, GSO Viet Nam 2016.
14. Nam Dinh Statistics Office – Namdinh Statistical Yearbook, GSO Viet Nam 2016.
15. Ninh Binh Statistics Office – Ninhbinh Statistical Yearbook, GSO Viet Nam 2016.
16. JICA, Viet Nam Japan Sewage Seminar, Mar/9/2017.